Methodic Protocols & Materials

For everybody's guidance

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Content

A. In vitro

I. Cloning

a) Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a precise method for exponentially amplifying a fragment of DNA (from a mixture of DNA molecules) in vitro.

Standard PCR is a method that is used to amplify DNA sequences of various lengths. A thermostable polymerase with proofreading functionality starts to amplify the DNA when the primers have bound to the respective binding site on the template DNA. The double-stranded DNA is denatured by heat, the primers are allowed to anneal and amplification is performed by the polymerase subsequently. These steps are performed repeatedly to amplify the DNA exponentially.

Touchdown PCR is a variation of Standard PCR. The annealing temperature for the primers is not constant during the PCR but decreases by 1 degree per cycle in the first cycles in order to avoid unspecific primer binding.

Mutagenesis PCR is used in order to introduce point mutations, insertions or deletions into DNA sequences of interest. For this purpose, specific primers are designed.

Reaction mix

- DNA template 1.0 ng – 10 ng
- Forward primer (10 µM) 1.0 µl
- Reverse primer (10 µM) 1.0 µl
- dNTPs (10 mM) 1.0 µl
- Polymerase 0.5 µl
- Polymerase-Buffer 5.0 µl
- $MgCl_2$
- DMSO
- ddH_2O fill up to 50.0 µl

Procedure

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95 °C	1:00 – 2:00 min.	1 cycle
Denaturation	95 °C	0:30 – 1:00 min.	
Annealing*	42 – 65 °C	0:30 min.	25 – 35 cycles
Extension**	72 – 74 °C	0:30 – 4:00 min.	
Final Extension	72 – 74 °C	5:00 min.	1 cycle
Hold	4 – 8 °C	Indefinite	1 cycle

* The annealing temperature for a specific amplification reaction will depend on the sequences of the two primers.

** Phusion: 15 – 30 sec/kbp Pfu: 1 – 2 min/kbp Q5: 20 – 30 sec/kbp

b) Circular Polymerase Extension Cloning (CPEC)

Circular Polymerase Extension Cloning (CPEC) is a method that is used in order to insert linear DNA sequences into linearized backbones. Moreover, it can be used to assemble various DNA fragments.

Procedure

1. Run a PCR as described in **I.** a)
2. Add 100 ng of the linearized vector backbone and equimolar amounts of the other assembly pieces to a 25 µl total volume assembly reaction mixture as follows:
    - linearized vector backbone (100 ng)
    - each additional assembly piece (to equimolar with backbone)
    - 5.00 µl 5 x HF Phusion Reaction Buffer
    - 1.00 µl 10 mM dNTPs
    - 0.75 µl DMSO
    - 0.50 µl 2 U/ml Phusion Polymerase
    - _____ µl dH_20 to 25 µl
3. Perform the assembly reaction in a thermocycler as follows:

	0:30 min	98 °C	1 cycle
	0:10 min	98 °C	
	0:30 min	55 °C	1 – 15 cycle(s)**
length* (kb) x	0:15 min	72 °C	
	10:00 min	72 °C	1 cycle

*The total length of the assembled product (in kb)
**The number of repeated cycles should exceed the number of assembly pieces

4. Transform 5 ml of the assembly reaction into 100 ml of competent *E. coli* and/or run an analytical agarose gel to check for successful assembly.

Source: https://j5.jbei.org/j5manual/pages/80.html

c) Restriction digest

In order to insert DNA fragments into plasmids via ligation, it is necessary to digest both components with restriction enzymes.

Procedure - Single DNA Digestion

The following is an example of a typical analytical single restriction enzyme digestion.

1. Add up the following:
    - 500 ng DNA
    - 5 µl 10 x appropriate NEBuffer
    - 1 µl restriction enzyme (10 U)
    - Fill up to 50 µl with nuclease free H_2O
2. Incubate for 30 minutes at 37°C
3. Heat inactivation: Incubate at 80 °C for 20 minutes

Larger or smaller scale DNA digestions can be accomplished by scaling this basic reaction proportionately.

Procedure – Multiple Restriction Enzyme Digests

Use the optimal buffer supplied with one enzyme if the activity of the second enzyme is acceptable in that same buffer (Check table supplied by NEB, BioBrick Assembly: Buffer 2.1). Follow the single restriction enzyme digestion by using 1 µl of the additional enzyme and take off 1 µl from the nuclease-free water.

d) Dephosphorylation

Antarctic Phosphatase catalyzes the removal of 5'-phosphate groups of DNA/RNA and thus prevents re-ligation of cut vectors. It is used before ligation.

Procedure

1. Reaction Mix
    - Restriction product
    - 1/10 of reaction end volume 10 x Antarctic Phosphatase Reaction Buffer
    - 1 µl of Antarctic Phosphatase
2. Incubate at 37 °C for 30 minutes
3. Heat inactivation: Incubate at 70 °C for 5 minutes
4. Continue with ligation

e) Phosphorylation

T4 Polynucleotide Kinase catalyzes 5'-phosphorylation of oligonucleotides. For ligation of two oligonucleotides, the relevant 5'-end needs to be phosphorylated. Primers are usually not phosphorylated during synthesis. For ligation of PCR-amplified oligonucleotides (for example blunt-end ligation of a linearized backbone) 5'-phosphorylation is necessary.

Procedure

1. Reaction mix (50 µl)
    DNA (up to 300 pmol of 5'ends)
    5 µl T4 Polynucleotide Kinase Buffer (10x)
    5 µl ATP (10 mM)
    10 units T4 Polynucleotide Kinase
    fill up to 50 µl water
2. Incubate at 37 °C for 30 minutes
3. Heat inactivation: Incubate at 65 °C for 20 minutes
4. Continue with ligation

Phosphorylation can also be performed with T4 DNA Ligase Buffer (the Addition of ATP is not necessary here).

f) DNA Ligation

DNA ligation is necessary to assemble digested DNA parts into a vector. The cut ends generated by restriction enzymes are put together by DNA ligase.

Procedure (20 µl batch)

1. Reaction mix
    - T4 Ligase Buffer 2 µl
    - T4 Ligase 1 µl
    - Digested Insert 6 µl
    - Digested Backbone 2 µl
      Molar ratio: 3 : 1 (Insert : Vector)
      For calculation: www.php.rsdnerf.com
    - add ddH_2O to 20 µl end volume
2. Incubate at 16 °C overnight or at room temperature for 30 minutes (results might be worse)
3. Inactivate at 65 °C for 10 minutes

II. Analysis and Purification

a) Agarose gel electrophoresis

Agarose gel electrophoresis is the most common used method to separate nucleic acids. Due to their negative charge DNA and RNA molecules can be moved through an agarose gel by an electric field (electrophoresis). Longer molecules move slower through the agarose matrix while short DNA fragments move faster and migrate further.

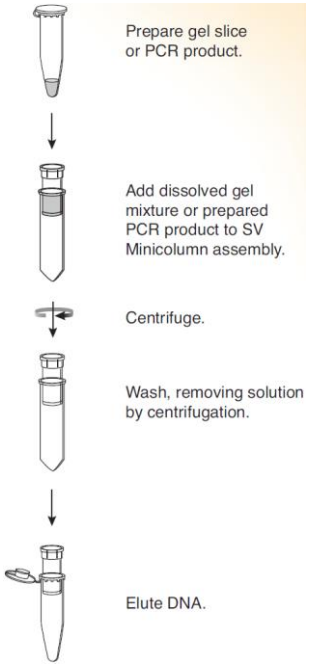
Procedure

In common we used 0.8 – 2 % agarose gels. Low concentrated gels lead to better results for large DNA fragments (2 – 6 kbp), while high concentrated gels lead to better results for small DNA fragments (0.3 – 0.7 kbp).

1. Mix desired amount of agarose with 1 x TAE-Buffer
2. Heat up liquid in microwave until whole agarose is dissolved
3. Let liquid cool down until you can touch the bottle with your hands
   Mix 2 µl Nancy-520 or HDGreen plus with 50 ml of agarose gel
4. Fill mixture into gel chamber and let it cool down (do not forget the well combs)
5. Fill-up chamber with 1 x TAE-Buffer
6. Take off well comb
7. Pipette 3 – 4 µl DNA ladder of choice into first pocket
8. Mix samples 5 : 1 with 6 x loading dye (5 µl sample with 1 µl loading dye) and pipette into pockets
9. Run electrophoresis with 120 V for 45 minutes.

b) Gel and PCR Clean-Up System (Wizard® SV)

Gel and PCR Clean-Up Systems are used to remove unincorporated primers, salts, and left-over dNTP´s from generated amplicons after PCR. This System also provides purification of amplicons from preparative agarose gel.



Procedure

Reference Wizard® SV Gel and PCR Clean-Up System Quick Protocol from Promega

Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5 ml microcentrifuge tube.
2. Add 10 µl Membrane Binding Solution per 10 mg of gel slice. Vortex and incubate at 50 – 65 °C until gel slice is completely dissolved.

B. Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing

4. Add 700 µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500 µl Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.
6. Empty the Collection Tube and re-centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

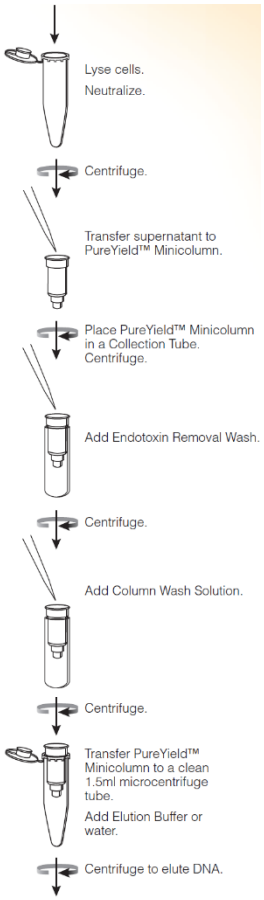
Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.
9. Discard Minicolumn and store DNA at 4 °C or - 20 °C.

Source:
https://www.promega.com/~/media/files/resources/protcards/wizard%20sv%20gel%20and%20pcr%20clean-up%20system%20quick%20protocol.pdf

c) Plasmid Miniprep (PureYield™ System)

To get the plasmid out of the cells, we use the PureYield Plasmid Miniprep System Kit from Promega. It is a fast way to purificate plasmid DNA out of a bacteria culture by cetrifugation.



Procedure

The whole purification was done according to the "Quick Protocol" of the PureYield Plasmid Miniprep System from Promega.

Prepare Lysate

1. Add 600 µl of bacterial culture to a 1.5 ml microcentrifuge tube.
   Note: For higher yields and purity use the alternative protocol below to harvest and process up to 3 ml of bacterial culture.
2. Add 100 µl of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.
3. Add 350 µl of cold (4 – 8 °C) Neutralization Solution, and mix thoroughly by inverting.
4. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.

5. Transfer the supernatant (~ 900 µl) to a PureYield™ Minicolumn without disturbing the cell debris pellet.
6. Place the Minicolumn into a Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 15 seconds.
7. Discard the flowthrough, and place the Minicolumn into the same Collection Tube.

Wash

8. Add 200 µl of Endotoxin Removal Wash (ERB) to the Minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds.
9. Add 400 µl of Column Wash Solution (CWC) to the Minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds.

Elute

10. Transfer the Minicolumn to a clean 1.5 ml microcentrifuge tube, then add 30 µl of Elution Buffer or nuclease-free water directly to the Minicolumn matrix. Let stand for 1 minute at room temperature.
11. Centrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, and store eluted plasmid DNA at – 20 °C.

Source:
https://www.promega.com/~/media/files/resources/protcards/pureyield%20plasmid%20miniprep%20system%20quick%20protocol.pdf

d) DNA Quantification with NanoDrop

NanoDrop is a UV-Vis spectrophotometer which can be used to measure quantity and purity of DNA in a sample using only 1 – 2 µl.

Procedure

1. Start the program for the NanoDrop and choose "Nucleic Acids"
2. Pipette 1 µl of a water sample onto the lower measurement and click “OK” for initializing
3. Load your blank and click "Blank"
4. Load your DNA samples and click "Measure"

The system calculates automatically the concentration in ng/µl, the 260/280 value and the 230/260 value.

e) Sequencing of DNA (Eurofins Genomics)

Sequencing is a method to control if you have your construct of interest in your plasmid. With or without mutations.

Procedure

1. Pipette 15 µl of template into 1.5 ml reaction tube, DNA concentration needs to be between 50 & 100 ng/µl. Dilute if necessary.
2. Add 2 µl primer (10 µM) of choice (do only use one primer!).

Premixed Samples (a mixture of template and primer)

- Templates should consist of 15 µl purified DNA with the concentration given above.
- Add 2 µl of primer with a concentration of 10 pmol/µl (10 µM).
- Ensure that the total volume of your premixed sample is 17 µl.

Optimum Primer Conditions

- Primers must not contain phosphorylation or fluorescent dyes
- The optimum primer length is between 16 – 25 bases
- The primer melting temperature (Tm) should be 50 – 62 °C
- The GC content of the primer should be 35 – 60 %
- Ideally one G or C should be located at the 3' primer end
- The number of 3' Gs or Cs should not exceed 2 Gs or Cs
- If possible, avoid >3 identical bases in a row in the sequence

Primer Concentration and Volume

- Exactly **10 pmol/µl** primer concentration is required per sequencing reaction
- Each primer must have a total **volume of 15 µl** (double distilled water or 5mM Tris-HCl); **5 µl of primer volume** is required for every additional sequencing reaction
- Concentration of primers with wobble bases must be calculated according to the following formula: $\mathbf{n^X}$ **x ConcPrimer**

n = number of bases within a wobble according to IUPC code, **X** = number of wobbles within the primer sequence. [e.g. 1 V (AGC) = 31 x 10 pmol/µl; 2 V (AGC) (AGC) = 32 x 10 pmol/µl]

Source:

http://www.eurofinsgenomics.eu/media/892645/samplesubmissionguide_valuereadtube.pdf

Address:

Eurofins Genomics | Anzinger Str. 7a | 85560 Ebersberg | Germany

f) SDS PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) is a widely used method to separate different proteins by mass.

Gels

	Separation gel (10 %)	**Stacking gel (4 %)**
MilliQ	1.975 µl	2.065 µl
Acrylamid/Bisacrylamid 37.5 : 1	1.670 µl	506 µl
Separation gel buffer	1.250 µl	
Stacking gel buffer		375 µl
SDS (10 %)	50 µl	30 µl
APS (10 %)	25 µl	15 µl
TEMED	2.5 µl	1.5 µl

Buffers

- Separation gel buffer (0.5 M Tris, 0.4% SDS, pH = 8.8)
- Stacking gel buffer (0.5 M Tris, 0.4% SDS, pH = 6.6)
- Running buffer (0.25 M Tris, 2 M glycine, 1% SDS , pH = 8.3)
- 2 x sample buffer “Laemmli-Buffer” (5 ml)

	Volume	**Final concentration**
Tris-HCL (1 M, pH 5.8)	650 µl	130 mM
SDS (10 %)	2000 µl	4 %
Glycerol (80 %)	1250 µl	20 %
β-Mercaptoethanol	500 µl	10 %
Bromphenolblue (pH = 6.75)	spatula tip	

- Staining buffer

	Volume/Amount
Coomassie brilliant blue G250	0.5 g
Coomassie brilliant blue R250	0.5 g
Methanol	100 ml
Acetate	20 ml
Aqua dest.	100 ml

Procedure

Load & run

1. Prepare separating gel and fill it into the chamber
2. Pour 1 ml isopropyl alcohol on the top of the gel to destroy air bubbles and prevent dehydration
3. Discard the isopropyl alcohol and pour the prepared stacking gel after separating gel is polymerized
4. Stick in the comb
5. If not used immediately, store the gel in wet cloth (to prevent dehydration) at 4°C
6. If used immediately, remove comb when the gel is fully polymerized and place it into SDS-PAGE chamber
7. Fill up chamber with running buffer
8. Heat up samples with 2 x sample buffer at 95 °C for 5 minutes and apply 20 µl to each pocket
9. Load one pocket with a commercial protein marker
10. Start the PAGE by applying 20 mA while stacking
11. Apply 40 mA while separation

Staining & washing of gel

1. Disconnect glass plates containing the already run gel
2. Cut off stacking gel
3. Put separation gel into staining buffer and shake at room temperature for at least one hour or heat it in the microwave
4. Put stained separation gel into distilled water and let shake for 10 minutes
5. Repeat previous step at least twice again with fresh distilled water

g) Protein clean up for Colicin E2 DNase domain

To test the functionality of our DNase domain, we established a protocol to clean it up to a degree that serves our needs

1. Grow 50ml of cells in erlenmeyer flask at 37 °C and 150 rpm until an OD of about 0.8 – 1 is reached
2. Transfer into 50 ml Falcon and centrifuge down at max speed for 10 min
3. Discard Supernatant
4. Freeze pellet in liquid nitrogen and transfer pellet into mortar
5. Lyse frozen pellet with a pestle
6. Resuspend lysed pellet in 3 ml of dissociation buffer
7. Incubate for one hout at RT for complete dissociation of Proteins
8. Centrifuge at max speed for 5 min to pellet precipitate
9. Transfer cleared supernatant to 10k MWCO centricon and centrifuge at 13,500 g for 20 min, discard flowthrough
10. Repeat step 9 until all clear lysate in dissociation buffer is used
11. Resuspend retentate in 1ml of Buffer and transfer to 1.5ml microreaction tube
12. You now have a lysate to test your protein.

B. In vivo

a) Chemically competent cells

The transformation of E. coli with plasmid DNA via heat shock transformation requires chemically competent cells.

Procedure

Day 1:

1. Grow Top10 / BL21 (DE3) overnight in 5 ml LB at 37 °C.

Day 2:

1. Inoculate 100 ml LB with 1 ml of saturated overnight culture of *E. coli* cells
2. Incubate at 37 °C and 150 rpm until an OD_{600} 0.4 – 0.6 (usually 2 – 3 h)
3. Incubate cells on ice for 5 minutes.

Note: After this point the cells should never touch anything that is warm – chill solutions, pipets, tubes, etc. beforehand.

4. Divide culture into 2 tubes with ~ 40 ml each
5. Centrifuge the culture at 4 °C and 3000 x g for 10 minutes
6. Gently resuspend each pellet with 15 ml of cold Mg^{2+}/Ca^{2+} solution (Do not vortex!).
7. Incubate in an ice bath for 30 minutes
8. Centrifuge the culture at 4 °C and 3000 x g for 10 minutes
9. Resuspend each pellet with 1.6 ml of cold 100 mM $CaCl_2$ solution
10. Incubate in an ice bath for 20 minutes
11. Combine cells to one tube
12. Add 0.5 ml cold 80 % glycerol and swirl to mix
13. Flash-freeze in liquid nitrogen as 100 µl aliquots
14. Store at - 80 °C.

Mixtures

- Mg^{2+}/Ca^{2+}
  - 3.25 g $MgCl_2 \cdot 6\ H_2O$
  - 0.6 g $CaCl_2 \cdot 2\ H_2O$
  - 200 ml ddH_2O
  - Autoclave
- $CaCl_2$ (100 mM)
  - 2.95 g CaCl2·2H2O
  - 200 ml dH2O
  - Autoclave

    Note: You can also make a 1:10 dilution of the 1 M stock

Source: http://openwetware.org/wiki/Griffitts:Chemocompetent_Cells

b) Bacterial cell culture

Bacterial cell culture is a method by which bacterial cells are cultivated under controlled conditions to multiply the number of cells.

Procedure

Starting culture: Under sterile conditions add about 5 ml of medium to a culture tube and insert the picked colony.

1. Cultivate the stock on agar plate e.g. until colonies grow (incubation usually at 37 °C)
2. Flame a glass pipette, open the bottle of medium and flame the mouth, measure out the amount you need to fill your tubes, flame the cap and recap the bottle as quickly as possible.
3. Remove the tube cap, flame the top of the culture tube, pipette in 5 ml, flame the top of the tube, and cap it. Pick a single colony (to assure the cells are from the same single clonal population) and transfer it to the medium by tapping a small (0.1 µl) pipette tip (held on a pipette) on the surface of the plate. Uncap a tube, flame the top, tip the tube so as to transfer cells from the pipette tip to the surface of the media without touching the inside of the tube with the non-sterile portion of the pipette, flame, cap.
4. Pipette the desired amount of antibiotic into each tube along the wall. Do not put the non-sterile part of the pipette inside the tube and use a new tip for each tube.
5. Vortex each tube for 1 – 2 seconds to mix well.
6. Take the tubes to incubate (usually at 37 °C) in an incubator or warm room.
7. Wait overnight or until your cells have reached the desired concentration.

Source: http://openwetware.org/wiki/Bacterial_cell_culture

c) Cell counting/plating

Cell counting/plating is a procedure to calculate the number of cells in a sample.

Procedure

1. Fill each tube in the dilution with 90 µl of LB.
2. Add 10 µl of the sample to the first tube and mix.
3. From the first tube, remove 10 µl and mix it in a second tube.
4. Repeat for the number of dilutions you wish to do (8 should be more than enough) [1].
5. Take 10 µl from each dilution and spread it out on an agar plate.
6. Allow droplet to dry and incubate.

The first dilutions will contain a thick lawn of cells and the last dilutions will contain no cells. There should be one drop which contains countable single colonies. From this, you can calculate the number of cells in the original sample. For example, if there 4 colonies on dilution 5, there are 4^4 cells/µl.

Source: http://openwetware.org/wiki/Bacterial_cell_culture

d) Heat Shock Transformation

Heat shock Transformation is a widely used technique to insert foreign plasmid DNA into chemically competent bacteria cells.

Procedure

1. Defrost stocks of competent cells (100 µl in 1.5 ml reaction tube) on ice.
2. Add DNA (2 – 6 µl) and incubate the suspension for 15 minutes on ice.
3. Heat shock is done by incubating the cells for 45 seconds at 42°C.
4. Put samples back on ice for 2 minutes.
5. Add 1 ml of LB medium and incubate for 1 hour at 37°C in order to obtain antibiotic resistance.
6. It might be useful to spin down cells at 5000 rpm for 5 minutes.
   Resuspend pellet in 100 µl LB.
7. Spread out cells on an agar plate

e) Colony PCR

Colony PCR is used to analyze whether a sequence of interest is present on a plasmid in E. coli. Flanking primers are used to amplify DNA in between the primer binding sites that are located on the plasmid backbone.

Procedure

The colony PCR is a modified PCR program employed to verify transformation success by amplifying the insert or the vector construct used for transformation. This is necessary due to the fact that a transformation with the empty vector may lead to antibiotic resistance.

1. Reaction mixture 1x - (25 µl)
   - 12.5 µl 2x Taq Master Mix
   - 0.5 µl VF2 (10 µM)
   - 0.5 µl VR (10 µM)
   - ddH_2O to 25 µl
2. Pick one colony with a sterile tip and suspend in reaction mixture
3. Start the PCR using the following program and 1 x mix.
4. Run a agarose gel to determine the product length

Procedure

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95 °C	5:00 min.	1 cycle
Denaturation	95 °C	0:10 min.	
Annealing	55 °C	0:15 min	30 cycles
Extension	72 °C	1 min/kbp	
Final Extension	72 °C	1.5 min/kbp	1 cycle
Hold	4 – 8 °C	Indefinite	1 cycle

f) Glycerol stock

In order to have a permanent culture of cells glycerol stocks can be made.

Procedure

1. Add 200 µl of sterilized glycerol or DMSO to 800 µl cell culture and mix well.
2. Shock freeze with liquid nitrogen
3. Store the stock at -80 °C.

g) Protein Expression (T7-promoter system)

(If working with different promotor system, use appropriate inducing chemical and take care of using appropriate strain)

Procedure (Choose volume appropriate to your desired amount of expressed protein)

1. Inoculation of 50 ml LB medium in a 100 ml flask with E. coli BL21 (DE3) strain transformed with desired plasmid
2. Incubation at 180 repulsion per minute (rpm) at 30 °C to an OD_{600} = 4
3. Transfer starter culture into 1 L LB medium in a 3 l flask resulting in an OD_{600} = 0.2
4. Incubation to an OD_{600} = 0.6 at 180 rpm and 30 °C.
5. Incubation for 15 minutes on ice.
6. Induction of protein expression with 20 ml of IPTG (stock concentration 1 M).
7. Incubation of the cell suspension over night at 180 rpm at 30 °C.

C. Materials

I. Buffers

a) Tris (1M, pH 7.5)

- 60.5 g Tris base
- adjust pH to 7.5 using 5 M HCl
- add ddH_2O to 500 ml
- store at room temperature

b) 1 x PBS

- 8.18 g NaCl (140 mM)
- 0.2 g KCl (2.7 mM)
- 1.77 g Na_2HPO_4 (10 mM)
- 0.24 g KH_2PO_4 (1.8 mM)
- Add. 1 l ddH_2O
- Adjust to pH 7.4 using HCl
- store at room temperature

c) TE buffer

- 10 mM Tris, adjust to pH 8.0 using HCl
- 1 mM EDTA (Ethylenediaminetetraacetic acid)
- store at room temperature

d) 50 x TAE

- 242 g Tris base
- add 57.1ml glacial acetic acid
- 100 ml of 500 mM EDTA (pH 8.0) solution
- add up to 1 L with H_2O
- mix Tris with stir bar to dissolve in about 600 ml of ddH_2O
- add EDTA and Acetic Acid
- bring final volume to 1 L with ddH_2O
- store at room temperature

e) Renaturing Buffer

- 0.02 M Tris HCl
- 0.02 M $MgSO_4$
- 0.08 M $NaCl_2$

f) Dissociation Buffer

- 6 M guanidine HCl
- 0.2 M NaCl
- 1 M K_2HPO_4 to acetify to pH 6.8

II. Media

a) 1.7-DYT (recipe for 500 ml)

- 2.5 g NaCl
- 8.0 g tryptone
- 5.0 g yeast extract
- add 5 M NaOH to adjust the pH at 7.0 (few drops will be enough)

b) LB (recipe for 1000 ml)

- 10 g tryptone
- 5 g yeast extract
- 10 g NaCl
- add 5 M NaOH to adjust the pH at 7.0 (few drops will be enough)
- for plates: add 15 g agar

c) SOB (recipe for 1000 ml)

- 0.186 g KCl
- 2.4 g $MgSO_4$
- 0.584 g NaCl
- 20 g tryptone
- 5 g yeast extract
- add 5 M NaOH to adjust the pH at 7.0 (few drops will be enough)

d) SOC (recipe for 1000 ml)

- 980 ml SOB
- 20 ml 1M glucose

Autoclave all media after mixing!

III. Stock solutions

a) Antibiotics

- **Ampicillin**
  1. Mix
     - 4 g ampicillin (100 mg/ml)
     - add 40 ml ddH_2O
  2. Sterile filtration
  3. Aliquot in 1 ml stocks and store at -20°C
  4. Use 1 µl per 1 ml medium

- **Chloramphenicol**
  1. Mix
     - 1 g chloramphenicol
     - add 40 ml ethanol
  2. Aliquot in 1 ml stocks and store at -20°C
  3. Use 1 µl per 1 ml medium

- **Kanamycin**
  1. 1.Mix
     - 3 g kanamycin (75 mg/ml)
     - add 40ml ddH_2O
  2. Sterile filtration
  3. Aliquot in 1 ml stocks and store at -20°C
  4. Use 1 µl per 1 ml medium

b) Induction chemicals

IPTG (Isopropyl-beta-D-thiogalactopyranoside)

1. Dissolve 238 mg IPTG in 10 ml water
2. Store in 1 ml aliquots at -20°C

c) *O*-methyl tyrosine

1. Dissolve 10 mg *O*-methyl tyrosine in 1 ml ddH_2O at 60 °C and 300 rpm.
2. Store stock solution at 4 °C

IV. Recipes

a) 9:25 Pfu MM (450 µl)

- 10x Pfu buffer (BSA) ..... 250 µl
- Pfu polymerase ..... 50 µl
- dNTPs (10 mM) ..... 50 µl
- MgCl2 (50 mM) ..... 50 µl
- DMSO ..... 50 µl

b) 2 x Taq MM (1250 µl)

- 10 x Taq buffer ..... 250 µl
- Taq polymerase ..... 25 µl
- 50 mM MgCl2 ..... 50 µl
- 10 mM dNTPs ..... 50 µl
- DMSO ..... 75 µl
- ddH2O ..... 800 µl

c) 10 x Pfu bufffer (100 ml)

- 200 mM Tris-HCl (pH 8.8 bei 25 °C) ..... 2.42 g
  100 mM $(NH_4)_2SO_4$ ..... 1.32 g
  100 mM KCl ..... 0.75 g
  20 mM $MgSO_4$ x 7 H_2O ..... 0.49 g
  1 % Triton X-100 ..... 1 ml
  ddH_2O .....up to 100 ml
- Autoclaving/sterile filtration after mixing.
- add 1 ml 100 mg/ml BSA (-> end conc. 1 mg/ml)

d) 10 x Taq buffer (100 ml)

- 500 mM KCl ..... 3.7 g
  0.8 % Triton X-100 ..... 800 µl
  100 mM Tris-HCl (pH 8.8 at 25 °C) .....up to 100 ml
- Autoclaving/sterile filtration after mixing.